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REGULAR ARTICLE

Separation and characterization of major milk proteins from Algerian Dromedary (*Camelus dromedarius*)

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Abstract

To characterize major protein fraction in Algerian dromedary's milk, two samples from Sahraoui population collected from two different regions were analyzed using electrophoretic and chromatographic techniques. Casein components and whey proteins were separated by DEAE-cellulose ion exchange chromatography and Sephacryl S200 permeation gel chromatography respectively, and then identified by polyacrylamide gel electrophoresis which looked different from the corresponding patterns of the caseins and whey proteins from cow milk. Differences in casein composition between camel and bovine milk may influence their digestibility, hydrolysis of camel milk caseins using four different proteases (trypsin, chymotrypsin, pepsin and papain) was studied. Caseins were more rapidly hydrolyzed by pepsin because of the greater number of potential pepsin cleavage sites present in the primary structures of camel caseins.

Key words: Camel milk, Enzymatic hydrolysis, Protein characterization

Introduction

The camel is one of the most important domesticated animals in the arid and semiarid zones of tropical and sub-tropical countries. Not only can camels survive under conditions of severe water and heat stress, but they also provide an important source of nutrients in desert communities, especially important during periods of prolonged drought (Farah, 1993). Available information concerning dromedary milk (Farah and Farah-Riesen, 1985; Beg et al., 1984, 1986a, b, 1987; Mehaia, 1987; Mohammed and Larsson-Raznikiewicz, 1989, 1991; Farah, 1993; Alim et al., 2005; Zhang et al., 2005; Konuspayeva et al., 2009; Al-Haj et al., 2010; Ereifej et al., 2011) is related mainly to the Arabian dromedary Camelus dromedaries species. The present work has been carried out in order to present a more description of the major milk proteins from Algerain dromedary's milk. This paper describes the separation of the caseins and the whey proteins by different chromatography and characterized by polyacrylamide gel electrophoresis. The sensitivity of camel's casein to the action of four proteases (trypsin, chymotrypsin, pepsin and papain) has been reported in the present work.

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Materials and Methods

Preparation of milk samples

Two samples of dromedary milk from *Sahraoui* type were collected in Ouargla and Ghardaia regions. They were defatted by centrifugation 4000g at 4°C for 15 min.

Separation of the protein fraction into caseins and whey proteins was conducted according to a modified method from Ochirkhuyag et al. (2000). Specifically, whole casein was obtained from skimmed milk by isoelectric precipitation (pH 4.3) at 22°C using 1N HCl. The precipitate was washed twice with distilled water at pH 4.3, solubilized at pH 7 by addition of 1M NaOH, precipitated again at pH 4.3 with 1N HCl and washed three times with distilled water. Finally, the whole casein was solubilized at pH 7, freeze–dried and stored at -20°C. The supernatant, containing the whey proteins was dialyzed against distilled water and then freeze dried and kept at -20°C until used.

Fractionation of caseins and whey proteins

The individual caseins were separated by ion-exchange chromatography on DEAE-Cellulose (DE52, Watman, France) column (26 mm i.d x 26 cm) equilibrated with 10 mM imidazole/HCl buffer, pH 7.0, containing 3.3 mM urea and 10 mM 2-mercaptoethanol, and the bound proteins were eluted from the column with a linear gradient of 0-1M NaCl, at room temperature, at a flow of 0.3 mL min^{-1} (Larsson-Raznikiewicz and Mohamed, 1986). The ion exchange chromatography was carried out on a low-pressure chromatography system (Bio-Rad, France). Fractionation of the whey proteins was performed by gel permeation chromatography on Sephacryl S200 (Amersham Biosciences) equilibrated with 0.02M Tris-HCl buffer pH 8.6 at room temperature, at a flow of 0.3 mL min^{-1}. The fraction size of collected eluate was about 1 mL. The absorbance of the fractions was determined at 280 nm. The fraction absorbance was plotted against elution volume using the LP Data View software.

Electrophoresis

Native PAGE with the vertical slab gel unit SE-250 series (Hoefer Scientific Instruments, San. Francisco) according to Hillier (1976) with a 12% (w/v) polyacrylamide gel in 0.75M Tris-HCl buffer, pH 8.9. Samples (2 mg mL^{-1}) were solubilised in 75 mM Tris-HCl buffer, pH 8.9, containing 10% (v/v) glycerol, and 0.01% (w/v) bromophenol blue.

Urea-PAGE was performed according to Andrews (1983) with an 8.2% (v/v) polyacrylamide gel in 75 mM Tris-HCl buffer, pH 8.9, in the presence of 4M urea. Samples (2 mg mL^{-1}) were solubilised in 75mM Tris-HCl buffer, pH 8.9, containing 4M urea, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.01% (w/v) bromophenol blue.

SDS-PAGE was performed on a 4.9% (w/v) polyacrylamide in 0.125M Tris-HCl buffer, pH 6.8 stacking gel and a 15.4% (w/v) polyacrylamide in 0.38M Tris-HCl buffer, pH 8.8 containing 0.1% (w/v) SDS separation gel (Laemmli and Favre, 1973). Samples were dissolved at 2 mg mL^{-1} in 0.125M Tris-HCl buffer, pH 6.8, containing 0.1% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.01% (w/v) bromophenol blue. After heating at 100°C for 3 min, 20 µL of sample was loaded in the gel. The molecular mass standards (Sigma chemical CO, Missouri, USA) were Urease (270.0 kDa), bovine serum albumin (66.0 kDa), egg albumin (45.0 kDa), carbonic anhydrase (29.0 kDa) and bovine lactalbumin (14.0 kDa). For both electrophoretic methods, volumes of 20 µL of samples were loaded in the gel,

proteins were fixed with 12% (w/v) trichloroacetic acid (TCA) for 30 min and then, stained for 60 min with 0.5% (w/v) R-250 Coomassie blue dissolved in a mixture of 50% (v/v) ethanol and 12% (w/v) TCA, followed by an overnight destaining in a solution containing 30% (v/v) ethanol, 7.5% (v/v) acetic acid, and 5% (w/v) TCA.

***In vitro* proteolysis of camel whole caseins**

The in vitro hydrolysis were performed as follows: a) chymotrypsin (EC 3.4.21.1; activity 45 U mg^{-1} protein) and trypsin (EC 3.4.21.4; activity 13500 U mg^{-1} protein): enzyme/protein ration 1/200 (w/w) in 0.1M sodium phosphate buffer (pH 8) at 40°C; b) pepsin (EC 3.4.23.1; activity 400-800 U mg^{-1}): enzyme/protein ration 1/250 (w/w) in 0.01N HCl (pH 2) at 37°C; c) papain (EC 3.4.22.2; activity 12 U mg^{-1}): enzyme/protein ration 1/800 (w/w) in 0.5M Tris-HCl buffer (pH 7) at 37°C and the final concentration of caseins was always 10 mg mL^{-1}. The reaction was stopped at different times by diluting the digestion mixture with the same volume of sample buffer (0.125M Tris-HCl buffer, pH 6.8 containing 0.1% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.01% (w/v) bromophenol blue, and then heating for 10 min at 100°C. Controls containing whole casein but without addition of enzymes, was also sampled.

Protein assay

The protein concentration was measured using Lowry's method with bovine serum albumin (BSA) as standard (Lowry et al., 1951); each measurement was done three times.

Results and Discussion

Electrophoretic and chromatographic separation of camel whey proteins

Samples of camel milk whey proteins from two different regions as well as cow milk were examined to determine whether they present differences or have similar composition as proteins from bovine milk. In order to identify the different whey proteins in camel and bovine milk, native-PAGE electrophoresis of whey camel samples from the two regions were compared to bovine whey proteins. In Figure 1 lane 1 (bovine whey) Ig, BSA, α-lactalbumin and β-lactoglobulin were observed. Several faint bands probably correspond to α-lactalbumin dimers and β-lactoglobulin octamers (Merin et al., 2001). Lane 2 and 3 are camel whey. Similar band to BSA and α-lactalbumin was observed. This result showed that α-lactalbumin

can exist in two forms, as seen previously in milk of Camelus dromedarius (Conti et al., 1985), with a slight difference in their amino acid composition and isoelectric point (5.1 and 5.3 respectively). Conti et al. (1985) reported that the two forms of α-lactalbumin in Camelus dromedarius whey differed at the first N-terminal position. The presence of small amount of a third α-lactalbumin was also reported by Ochirkhuyag et al (1998). β-lactoglobulin appears only in bovine milk, which is in agreement with published data (Farah, 1986; Ochirkhuyag et al., 1998). Lack of β-lactoglobulin is also reported for milk of other species including human milk (Jenness, 1985) and is not due to preparation artifact, science in the SDS-PAGE electrophoresis (Figure 2) no band in the vicinity of 18 kDa was detected in camel whey.

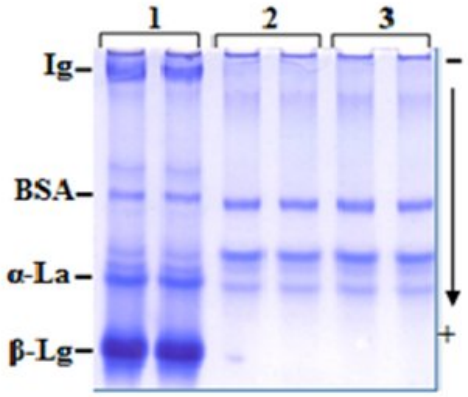

Figure 1. Native-PAGE of camel and bovine whey proteins.
(1) Bovine whey, (2), (3) camel whey from Ouargla and Ghardaia respectively.

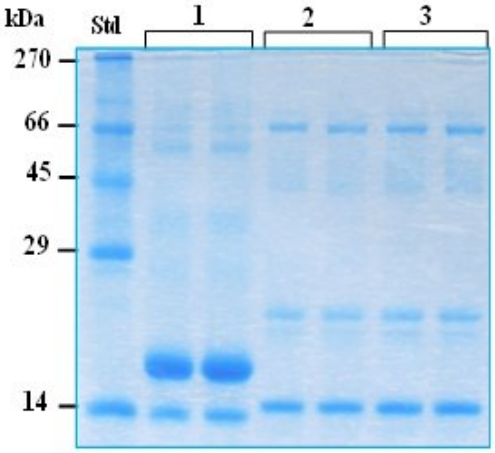

Figure 2. SDS-PAGE of camel and bovine whey proteins.
(1) Bovine whey, (2), (3) camel whey from Ouargla and Ghardaia respectively, std: molecular weight standard.

SDS-PAGE of cow and camel milk whey proteins (Figure 2) showed four bands in the two camel milk samples. Their MWs were estimated at 66.0, 43.0, 29.0 and 14.0. Two major bands (66.0 and 14.0) were identified as serum albumin and α-lactalbumin by comparing the observed electrophoretic profiles with those reported by Ochirkhuyag et al. (1998). The two forms of α-lactalbumin have identical molecular weight according to their mobility in SDS-PAGE at pH 8.0.

A chromatogram of camel whey is presented in Figure 3. Camel whey proteins were separated into 3 fractions. As observed by native-PAGE (Figure 4), serum albumin was eluted in fraction1, the two forms of α-lactalbumin were eluted in fraction 2 and the third peak contained no identified proteins which could correspond to heterogeneous camel milk whey proteins (Beg et al., 1987). It is assumed that other whey components such as lactoferrine (75-76 kDa), lactoperoxydase (69 kDa) and the 43 kDa fraction (Kappeler, 1998) will not be separated on the columns used in this work. It would be reasonable to assume that the whey proteins of camel and bovine that appear at similar elution times are actually identical as was presented by sequencing of the different camel whey proteins by Ochirkhuyag et al. (1998).

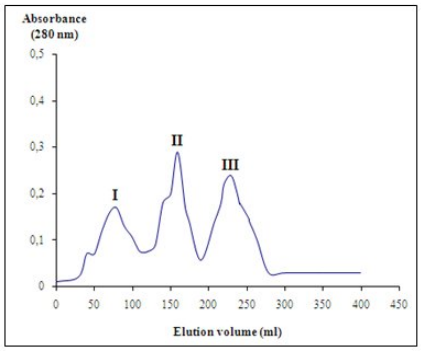

Figure 3. Elution pattern of permeation chromatography on sephacryl S200 of camel whey proteins. The column was equilibrated in 0.02M Tris-HCl buffer pH 8.6. the flow rate was of 0.3 mL min^{-1}. The fraction size was 1 mL.

Electrophoretic and chromatographic separation of camel milk caseins

Characterization of cow and camel milk caseins were performed by urea-PAGE (Figure 5) and the electrophoretic patterns show the same main bands of equal intensity and mobility for the two camel milk samples from different regions. The electrophoretic pattern showed two sharp and distinguishable main bands in camel milk.

According to their increasing electrophoretic mobility, in comparison with cow milk casein, the two bands can be regarded as a possible homologue to bovine. The last band of cow milk sample which corresponding to α_S doesn't appear in the whole camel casein fraction. No protein bands homologous to bovine k-casein could clearly be detected in the electrophoretic pattern. Compared with cow milk caseins, camel's casein presented a lower mobility, than that of their bovine counterparts. This is probably depending on the degree of their phosphorylation (Mohamed and Larsson-Raznikiewicz, 1991). Mohamed and Larsson-Raznikiewicz (1991) and Ochirkhuyag et al. (1997) have obtained dromedary β-like casein band with a migration similar to that of cow k-casein. Neither a band corresponding to κ–casein, nor proteins with mobility similar to bovine casein fractions could be detected. SDS dissociates proteins into their constituent polypeptide chains and has been used for the separation of the proteins according to their molecular weight. SDS-PAGE patterns of camel caseins are presented in Figure 6. The marker proteins with molecular weight between 14 200 and 270 000 were excellently separated in the selected acrylamide gel concentration (15.4%). the molecular masses of the camel casein bands estimated from calibration curve, are 32 000 and 35 500. This is considerably higher than the possible homologous bovine caseins which are estimated at 24 000 for β-casein and 22 000 to 27 000 for α_S-casein.

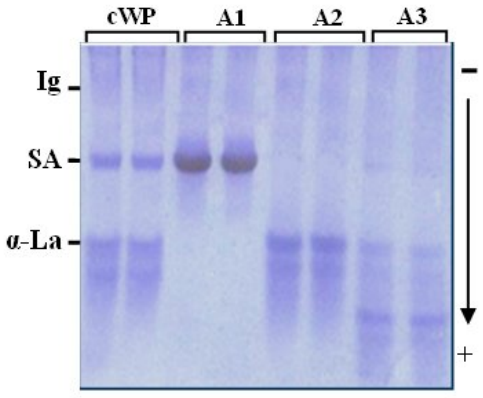

Figure 4. Native-PAGE patterns of fractions issued from permeation chromatography on Sephacryl S200 of camel milk whey proteins.
cWP : camel whey proteins ; A1, A2 and A3: whey protein fractions.

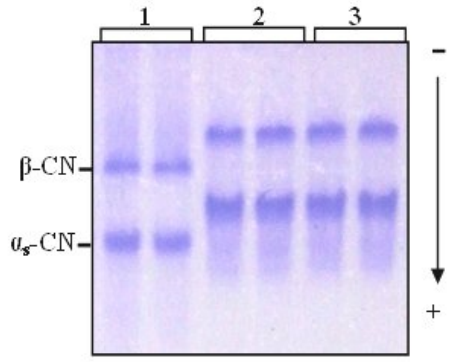

Figure 5. Urea-PAGE of camel and bovine casein.
(1) Bovine casein, (2), (3) casein from Ouargla and Ghardaia camel milks.

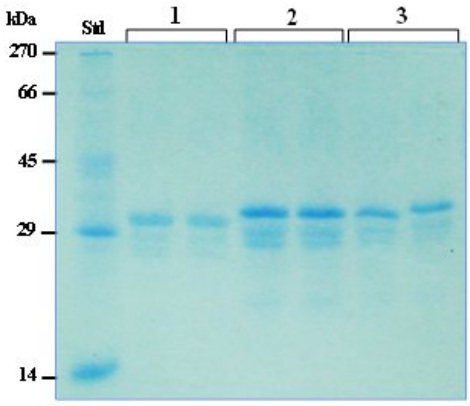

Figure 6. SDS-PAGE of camel bovine casein.
(1) Bovine casein, (2), (3) casein from Ouargla and Ghardaia camel milks, std: molecular weight standard.

Acid-precipitated of whole casein from dromedary milk were separated by anion-exchange chromatography on DEAE-cellulose column (Figure 7). As already know, classical anion exchange chromatography on DEAE-cellulose column resolved whole bovine casein into different fractions containing γ, k, β, α_{S2} α_{S1} respectively (Mercier et al., 1968). As shown in figure 7 caseins were eluted in four peaks at 0.08, 0.16, 0.23 and 0.26 mol L^{-1} NaCl respectively. The electrophoretic pattern of each peak obtained by anion exchange chromatography (Figure 8) suggest that peak 1 contained β-, peak 2 and 3 contained α_{S1}- and peak 4 contained α_{S2}-casein which was co-eluted with α_{S1}-caseins. In comparison to the results from Kappeler et al. (1998), in which κ-casein was eluted faster than other caseins, the most prominent finding is the absence of a chromatographic peak referable to κ-casein. It may therefore be difficult to fractionate them in a single run of anion-exchange in which proteins are fractionated based on their charge.

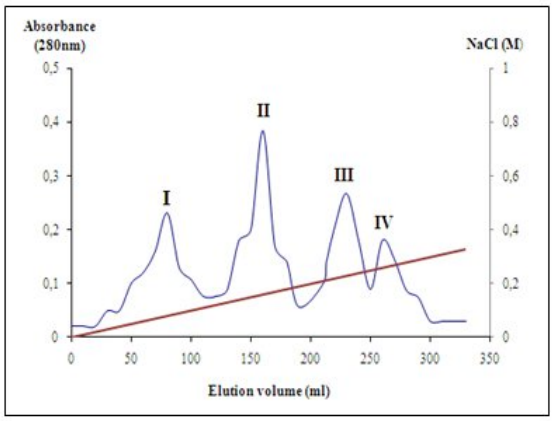

Figure 7. Elution pattern of ion exchange chromatography on DEAE cellulose of camel casein. The column was equilibrated in 10mM imidazole/HCl buffer, pH 7.0, containing 3.3 mM urea and 10mM 2-mercaptoethanol, eluted with a linear gradient of 0-1 M NaCl, at room temperature, at a flow of 0.3mL min^{-1}.

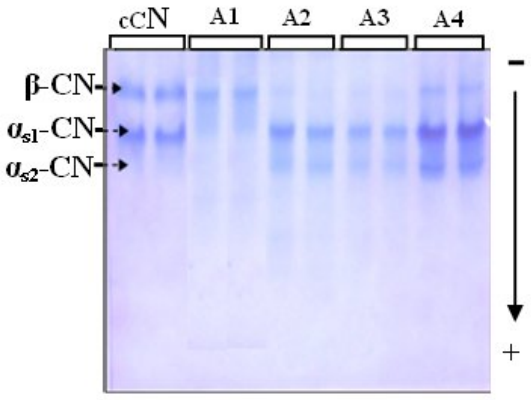

Figure 8. Urea-PAGE patterns of fractions issued from ion exchange chromatography on DEAE cellulose of camel milk caseins.
cCN: camel whole casein; A1, A2, A3 and A4: casein fractions eluted at 0.08, 0.16. 0.23 and 0.26 mol L-1 NaCl respectively.

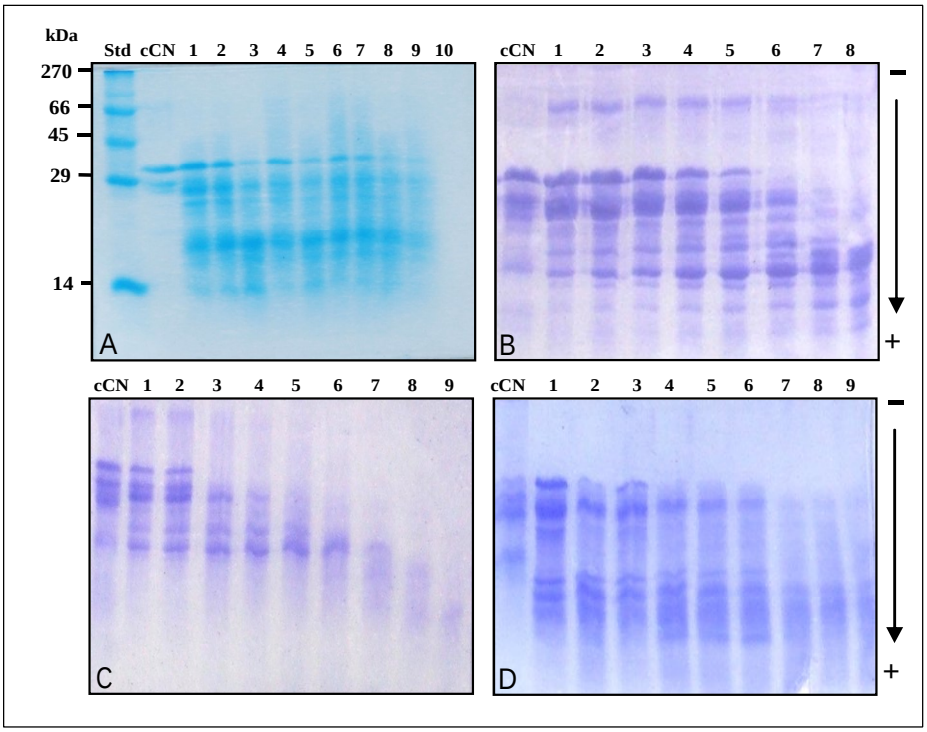

Figure 9. SDS-PAGE analysis of the kinetics of chymotrypsin (A), trypsin (B), pepsin (C) and papain (D) hydrolysis of camel whole casein. Std: molecular weight standard; cCN : camel whole casein, 1,2,3,4,5,6,7,8,9: Hydrolysis times at 5, 10, 15, 20, 30, 45,60, 120, 180 min.

Enzymatic hydrolysis of whole camel casein

In order to study the degree of hydrolysis of camel milk caseins, the enzyme-treated and untreated protein samples of whole CNs were analyzed by SDS-PAGE for pepsin, trypsin, chymotrypsin and papain assays (Figure 9). The α_{S1}-CN was almost fully degraded by both enzymes after 10 min of incubation; it appears like sharp and diffuse band; whereas hydrolysis of β-CN was complete after 5 min of hydrolysis by pepsin, 30 min by trypsin and papain and 48h by chymotrypsin. β-CN from camel milk were more

resistant to trypsin, chymotrypsin and papain digestion, it's very quickly hydrolyzed by pepsin. After 5 min of hydrolysis of camel CNs by chymotrypsin, trypsin and papain, some peptide fragments were still detected on SDS-PAGE, which were stable up to 4h of incubation with chymotrypsin, trypsin and papain, but whith pepsin, peptide fragments were disappeared completely after 60 min of incubation. Similar peptide fragments were not obtained when CNs were treated with different proteases. The major protein components of camel milk, α_{S1}- and β-CNs, contain different numbers of covalently attached phosphate groups bound to residues of serine and threonine (Dickson and Perkins, 1971; Medina et al., 1992). The bound phosphate groups influence many functional properties of these proteins, including their digestibility, bioavailability of divalent cations and immunogenicity (Tezcucano et al., 2007). As reported previously, covalently bound phosphate groups of CNs are supposed to be one of the factors reducing the digestibility of CNs (Li Chan and Nakai, 1989). Taking into account the number of phosphoseryl and phosphothreonyl residues as one of the possible factors reducing the hydrolysis of CNs, β-CN should be hydrolyzed to a greater extent than α_{S1}-CN in both animal species. The results obtained on SDS-PAGE (Figure 9) showed that this was not the case when trypsin or chymotrypsin was used. At least, some portion of β-CN of both species treated by trypsin or chymotrypsin remained uncleaved even after 15 min of digestion. It seems that this protein, which contains four phosphoseryl residues, could better resist digestion by trypsin or chymotrypsin than bovine α_{S1}-CN containing eight phosphate groups (Salami et al., 2008). Thus, another factor must be taken into account to explain the greater hydrolysis of α_{S1}-CN by trypsin, chymotrypsin or papain compared with β-CN, which could be the number of target peptide bonds available for attack by the proteases. Although the greater susceptibility of α_{S1}-CN to trypsin, chymotrypsin and papain hydrolysis compared with β-CN could arise from the number of enzymatic cleavage sites, the accessibility of these sites is another important factor.

Conclusion

Results of this study performed on Algerian dromedary's milk proteins showed homogeneity between samples under both quantitative and qualitative aspects. Results indicate that the whey of Algerian camel milk contains a major protein, α-lactalbumin, existing in two different forms, with identical molecular weight, which are eluted together during Sephacryl S200 permeation gel chromatography and migrated in the same region on SDS-PAGE. They could be separated by isoelectric focusing and/or by anion exchange chromatography, using column with high resolving power. β-lactoglobulin is responsible for some of the observed allergies to cow's milk. Since Algerian camel milk is devoid of β-lactoglobulin, it could be interesting as a new raw material for infant diet and for alleviating some allergic reactions, especially in children. Algerian camel milk samples contain three casein fractions (α_{S1}-, α_{S2}-, β-caseins). Interestingly, analytical results suggested the absence of κ-casein in two samples. The observed differences of their molecular masses, in comparison with cow milk casein, are either due to few variations in their primary sequences or to the divergence of their primary glycosylation and/or phosphorylation.

Whole camel milk caseins were used as substrate for different proteases such as trypsin, chymotrypsin, pepsin and papain. These digestive enzymes, which have different specificities towards the protein substrates, have generated different peptides during proteolysis. The kinetic degradation patterns of camel caseins were visualized by SDS-PAGE. The extent of proteolysis of camel milk caseins by different enzymes depended on the number of target sites available for each enzyme and perhaps on how well these target peptide bonds are accessible to the proteases. The accessibility of the target sites to the digestive enzymes finally depends on fine tertiary structure of milk proteins. Nonetheless, investigations at DNA level are necessary in order to better characterize dromedary's genetic structure.

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